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ORIGINAL INVESTIGATION

Mucosal innate response stimulation induced by lipopolysaccharide protects against *Bordetella pertussis* colonization

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Abstract Non-specific enhancement of the airways innate response has been shown to impair lung infections in several models of infection such diverse as influenza A, *Streptococcus pneumoniae,* and *Aspergillus niger*. Our aim was to evaluate whether a similar event could operate in the context of *Bordetella pertussis* respiratory infection, not only to enrich the knowledge of host–bacteria interaction but also to establish immunological basis for the development of new control strategies against the pathogen. Using a *B. pertussis* intranasal infection model and coadministration of different TLR agonists at the moment of the infection, we observed that the enhancement of innate response activation, in a TLR4-dependent way, could efficiently impair *B. pertussis* colonization ($P < 0.001$). While LPS from different microbial sources were equally effective in promoting this effect, flagellin and poly I:C coadministration, in spite of inducing expression of innate response markers TNFα, CXCL2, CXCL10 and IL6, was not effective to prevent *B. pertussis* colonization. Our results indicate that during the early stage of infection, specific anti-microbial mechanisms triggered by TLR4 stimulation are able to impair *B. pertussis* colonization. These findings could complement our current view of the role of TLR4-dependent processes that contribute to anti-pertussis immunity.

Keywords *Bordetella pertussis* · Stimulated innate resistance · TLR4

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Introduction

Pertussis or whooping cough is an acute respiratory tract infection caused by *Bordetella pertussis*, a fastidious Gram-negative bacterium. This disease, though preventable by vaccination, remains one of the top ten causes of death worldwide in childhood by infectious diseases [9, 16, 21]. Though the reasons for this persistence or resurgence of pertussis are still not fully elucidated [9], it would be important to deepen the understanding of some aspects of the infection and the factors affecting the infectious process since this knowledge is expected to contribute in the designing of new strategies to improve the control of the disease.

Recently, a protective phenomenon termed stimulated innate resistance (StIR) has been reported for different inhaled microbial pathogens [8]. This phenomenon consists in a stimulation of lung innate immune mechanisms with an aerosolized bacterial lysate that confers a high level of protection against challenge with otherwise lethal inocula for a short term [6]. This protective effect was recently shown to be operative for different bacterial pathogens and even to other microbial classes such as virus and fungi [8, 22]. In order to gain insight in the relevance of innate mechanisms of clearance of *B. pertussis* infection, we tested the occurrence of StIR-like phenomenon in a *B. pertussis* infection model by performing mice infections upon airway stimulation with different pathogen-associated molecular patterns

(PAMPs). We used agonist with well-known capacity to stimulate lung innate response such as bacterial lipopolysaccharide (LPS), Flagellin (FliC), and poly I:C. [15, 25]. We observed that when mice were treated with a suspension of *B. pertussis* supplemented with LPS, the number of bacteria recovered from the lung decreased drastically in comparison with those observed when *B. pertussis* suspension was used alone. This effect appears to be LPS dependent regardless of its source. Similar results were obtained with LPS from either *B. pertussis* or *B. bronchiseptica* or *Escherichia coli* or *Sinorhizobium meliloti*. These findings indicate the existence of TLR4-dependent mechanisms that contribute to host defense in the early stage of *B. pertussis* infection and complement previously described the role of TLR4-dependent effects in shaping anti-*B. pertussis* adaptive immunity [11].

Materials and methods

Bacterial strain and growth condition

B. pertussis strain used in this study was Tohama I (CIP 8,132, Collection de l'Institut Pasteur, France). *B. pertussis* strain was grown on Bordet-Gengou (BG) agar plates containing 10% defibrinated sheep blood. For LPS extraction, subcultures were grown in Stainer-Scholte liquid medium for 20 h at 36°C until the optical density measured at 650 nm reached 1.0 [12].

Endotoxin-free flagellin FliC was prepared from *Salmonella enterica serovar Typhimurium* ATCC 14,028 as previously described [19]. Poly I:C was from Invitrogen (USA) and *Escherichia coli* LPS was from Sigma.

Lipopolysaccharide extraction

The LPS from either *B. pertussis, B. bronchiseptica* 9.73 or *Sinorhizobium meliloti* Rm41 was isolated by the hot phenol-water method [24] along with the modifications previously described [12]. The obtained samples of LPS were dialyzed and lyophilized. Dry weight measures were used to determine the amounts of the LPS obtained. The quality of each sample was checked by SDS–polyacrylamide gel electrophoresis (SDS–PAGE, not shown) [14].

Mouse infection

All mouse procedures were performed in accordance with National Regulations. Three to four-week-old female BALB/c mice (Biol SAIC) and C3H/HeJ mice (originally from Jackson Laboratories, USA and maintained at BIOL SAIC) were used in our studies. Inocula were prepared, and mice were inoculated intranasally as previously described [17]. Mice were killed by cervical dislocation at 24 h after infection, and the lungs were removed and homogenized in 1 ml of sterile PBS. Appropriate dilutions were plated on BG blood agar plates and counted after 4 days of incubation at 37ºC to determine colony-forming units (CFU) per lung. A minimum of five mice per group were used in each of the four independent experiment performed. A Tukey test was used for statistical analysis.

Mouse weight gain test (MWG)

The MWG-test was carried out using groups of five BALB/c mice outbred mice (15–20 g) which were inoculated using intranasal route with a volume (40 µl) containing *B. pertussis* Tohama suspension (10^6 CFU of Bp) alone or with LPS (1 µg), or LPS (1 µg) alone. Control group received an equal volume of sterile PBS. Animals were observed for 7 days, and body weight was recorded after 16 h, 3 and 7 days. Inoculums were considered non-toxic when passing the following requirements (WHO and EP requirements): (a) the total weight of the mice from the vaccine group 3 days after treatment was the same or higher than the initial weight, (b) at the end of 7 days, the average weight gain of the vaccine group was not less than 60% of the control group and (c) not more than 5% of the animals died during the test period.

RNA preparation from lung tissue

Mice were killed by cervical dislocation, and the thoracic cavity was exposed. The two left lobes of the lung were removed, snap frozen in a dry ice/ethanol bath and stored at −80ºC until further use. RNA preparation from whole lung tissue was performed using the Illustra RNAspin kit (GE, USA). Samples were placed on ice, and quantification of RNA was performed using a ND-1,000 NanoDrop spectrophotometer at 260 nm. Measurements of A260/280 were used to determine the purity of the RNA.

cDNA synthesis

The synthesis of cDNA was performed with a Reverse Transcription System kit (Promega) according to the manufacturer's protocol using random primers. One microgram of RNA was used for each sample. The reaction was incubated at room temperature for 10 min, and reverse transcription was performed in a thermal cycler at 42ºC for 15 min and 95ºC for 5 min. Samples were placed on ice for 5 min to stop the reaction and diluted 1:10 in nuclease-free H_2O. Real-time PCR was performed using the SYBR green master mix 2× (Bio-Rad, USA). The following primers designed using Primer Express software (Applied Biosystems, USA) were employed: qmouCXCL2$_{fwd}$

CCCTCACAGGAAGAACCAAA, qmouCXCL2$_{rev}$ CACA TCGGTACGATCCAGGC; β-actin$_{fwd}$ CGTCATCCATG GCGAACTG, β-actin$_{rev}$ GCTTCTTTGCAGCTCCTTC GT; IL6$_{fwd}$ GTTCTCTGGGAAATCGTGGAAA, IL6$_{rev}$ AAGTGCATCATCGTTGTTCATACA; TNFα_{fwd} CATC TTCTCAAAATTCGAGTGACAA, TNFα_{rev} CCTCCA CTTGGTGGTTTGCT;.and qmouCXCL10$_{fwd}$ GGAGTGA AGCCACGCACAC, qmouCXCL10$_{rev}$ TGATGGAGAGA GGCTCTCTGC. All samples, including a negative control using H_2O instead of cDNA, were run in duplicates. The data were analyzed using ΔCt calculations using β-actin expression levels as normalizer as previously described [19]. Results were expressed as fold increase over values from PBS-treated mice.

Results

Protection against *B. pertussis* colonization

It has been shown that profound inflammatory lung responses to a bacterial lysate improved the survival of mice infected with different bacteria [6, 8]. Using a similar approach, we investigated the effect of stimulation of lung mucosal innate immunity on *B. pertussis* colonization. In order to evaluate this, animal assays using intranasal *B. pertussis* infection were performed. In particular, the effect on colonization after the coadministration of *B. pertussis* Tohama suspension (10^6 CFU of Bp) with different agonist such as LPS (1 μg), flagellin (1 μg) and poly I:C (50 μg) was analyzed. The results obtained were compared with those obtained in mice inoculated with *B. pertussis* Tohama alone (Bp alone). Twenty-four hours after infection, the lungs were collected for bacterial count. The results obtained showed significant differences between animals challenge with Bp alone and the group infected with Bp + LPS$_{Bp}$ ($P < 0.001$; Fig. 1). While in mice inoculated with Bp, the number of recovered colonies from lungs 24 h after challenge was similar to those found with Bp + poly I:C; in mice treated with Bp + LPS$_{Bp}$, the number of lung recovered colonies at 24 h dropped at least two orders of magnitude with respect to the counting in Bp alone and Bp + poly I:C treatments (Fig. 1). The number of the recovered CFU from the lungs of Bp + FliC infected mice was slightly inferior to that observed with Bp alone.

Similar experiments were performed in C3H/HeN and its derivative TLR4-defective strain (C3H/HeJ mice strain). As shown in Fig. 2 (Panel A), the results obtained in C3H/HeN were similar to those obtained in BALB/c, being the LPS treatment the only one that was able to reduce significantly the number of recovered colonies from infected mice. As observed in BALB/c, a slight reduction in pulmonary bacterial burden was observed with flagellin treatment

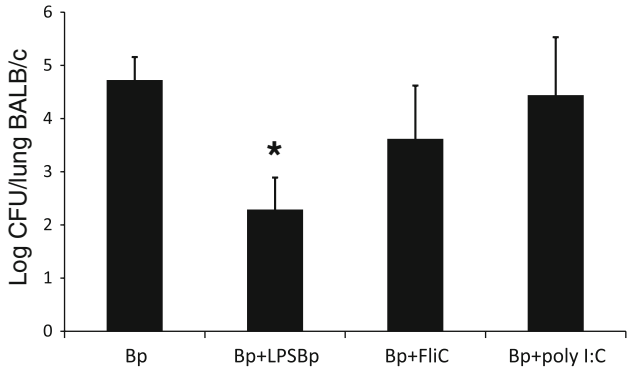

Fig. 1 Bacterial colonization upon coadministration with different TLR agonists. Nasal challenge of mice was performed with sublethal dose (10^6 CFU 40 μl^{-1}) of *B. pertussis* Tohama strain (Bp) either alone (Bp) or supplemented with 1 μg of either purified *B. pertussis* LPS (Bp + LPS$_{Bp}$). flagellin (Bp + FliC) or 50 μg of poly I:C (Bp + poly I:C). Lungs of infected mice were collected for bacterial counts 24 h after the challenge. The lungs aseptically removed, homogenized in the sterile PBS, were serially diluted and then plated on Bordet–Gengou plates supplemented with defibrinated sheep blood to determine bacterial recoveries at the time point analyzed. Results show the average counts of five mice per group. Results are representative of at least four independent experiments. *$P < 0.001$ in Tukey Test

for C3H/HeN strain. Interestingly, the number of bacteria recovered at 24 h from the lungs of C3H/HeJ mice either treated with Bp, Bp + LPS$_{Bp}$ or treated with Bp + poly I:C was similar (Fig. 2, panel A). Again, with flagellin treatment, a slight reduction in lungs bacteria counts was observed for C3H/HeJ strain (Fig. 2, Panel A). Using a higher dose of flagellin (20 μg), an impact on the number of recovered bacteria from C3H/HeJ lungs was observed (Fig. 2, panel B). However, this effect was much weaker than the one caused by LPS. Experiments performed using the LPS from either *B. pertussis, E. coli, S. meliloti* or *B. bronchiseptica* showed that effect appears to be LPS dependent regardless of its source (Fig. 3).

Gene expression analysis

Lung expression of several cytokine-encoding genes was highly induced upon the infection in the presence of different TLR agonists (Fig. 4). CXCL2 and TNFα values obtained for the LPS treatment were superior compared to other treatments. The levels of IL6 induced by FliC treatments were superior compared to the other conditions, whereas for CXCL10, all agonists induced higher expression than *B. pertussis* infection alone. In this case, although there is a tendency of higher induction in the case of LPS addition, no significant differences were detected.

Mouse weight gain test

This test is usually employed to measure the toxicity of pertussis formulations. Results of mouse weight gain test

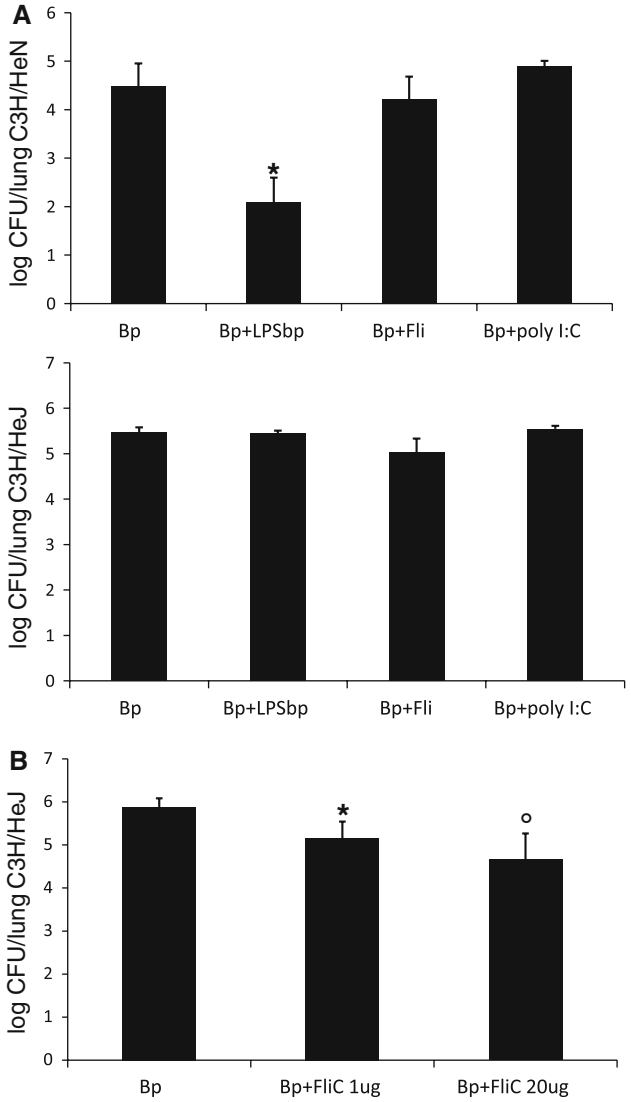

Fig. 2 Impairment of *B. pertussis* infection is TLR4 dependent. *Panel A* Bacterial infection was performed in similar conditions as reported in Fig. 1, using either TLR4-defective C3H/HeJ or their isogenic TLR4 competent C3H/HeN strain as control. Results show the average bacterial counting of five mice per group. $*P < 0.001$ in Tukey Test. *Panel B* Nasal challenge of C3H/HeJ mice was performed with a similar sublethal dose of *B. pertussis* (Bp) than previous experiments, either alone (Bp) or supplemented with 1 or 20 μg of purified flagellin. Results show the average of the number of recovered bacteria at the time point analyzed (24 h). Results are representative of at least two independent experiments. $*P < 0.01$ and $^{O}P < 0.001$ in Tukey Test, when compared to Bp infected control

obtained with Bp + LPS were compared with those of Bp or LPS treatments as well as with PBS. Paired sample testing revealed the weight loss of LPS- and Bp + LPS-treated mice at 3 days post inoculation. Weight gains at 7 days were 100,7 ± 3,7%, 94,1 ± 3,3%, 100,1 ± 0,3% and 100,7 ± 3,7% for LPS, Bp, Bp + LPS and control groups, respectively. As expected, LPS induced toxicity; however, its addition to Bp suspension did not increase disease severity.

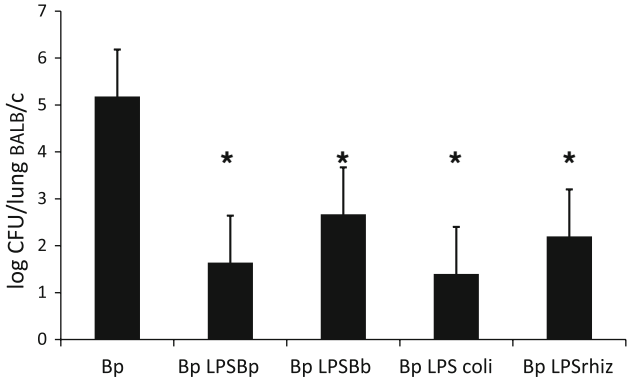

Fig. 3 Effects of coadministration of LPS from different microorganisms. BALB/c mice were infected and processed as described for Fig. 1. At the moment of the infection, LPS from different sources were included as follows: *B. pertussis* (Bp + LPSBp), *B. bronchiseptica* (Bp + LPSBb), *Escherichia coli* (Bp + LPScoli) and of *Sinorhizobium meliloti* (Bp + LPSrhiz). Results show the average bacterial counting of five mice per group and are representative of at least two independent experiments. $*P < 0.001$ in Tukey Test

Discussion

The stimulated innate resistance (StIR) phenomenon is mediated by the enhancement of natural anti-microbial capacity of inducible innate response. This phenomenon has been reported for different inhaled microbial pathogens such as *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* among respiratory tract bacterial pathogens [8]. Although the mechanistic basis of this effect is incompletely understood, it has been proposed that it originated in the activation of several anti-microbial mechanisms in resident as well as in immune cells. These mechanisms include anti-microbial peptide production, enhancement of oxidative radical production and increase in phagocytic capacity of several cellular types [8]. Since *B. pertussis* is known to produce a myriad of cellular adhesins and toxins aiming to manipulate the early host response to achieve efficient colonization [4, 5], we tested the putative protective role of enhancement of innate response at the moment of pertussis infection, mimicking such StIR process. To this purpose, we coadministered different PAMPs namely LPS, flagellin and poly I:C at the moment of infection. The results obtained showed that in mice treated with Bp + LPS_{Bp}, the number of lung recovered colonies at 24 h dropped at least two orders of magnitude with respect to the counting in Bp alone and Bp + poly I:C treatments ($P < 0.001$; Fig. 1). These results showed that TLR4-dependent response was the major responsible of the observed effect. In order to test this hypothesis, experiments using mice unable to respond to LPS because they are defective in TLR4 function (C3H/HeJ mice strain) were performed. Using this system, we could confirm that *B. pertussis* clearance was completely

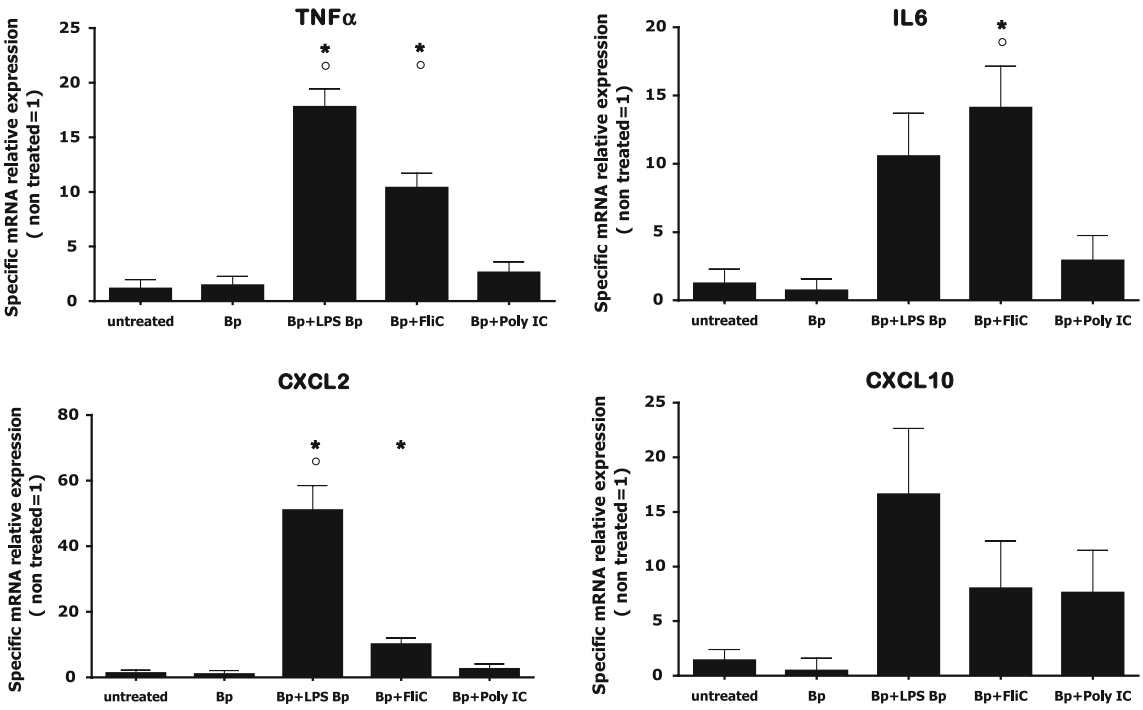

Fig. 4 Lung cytokine relative expression levels. Total RNA was extracted from lung, and relative expression of different cytokines evaluated by RT-qPCR. Fold increase values were referred to non-infected condition. Results show the average of three independent mice per group. Experiment was repeated three times. Results shown are from a representative single experiment. *$P < 0.01$ in Tukey Test, when compared to non-infected control. ^{O} indicate $P < 0.01$ in Tukey Test when compared to poly I:C treatment

dependent of TLR4 functionality (Fig. 2). Although higher doses of flagellin can affect the colonization of *B. pertussis*, the observed effects were much weaker than the ones caused by LPS, indicating differences in the effector mechanisms elicited by their receptors.

In spite of the differences in bacterial clearance among the different TLR agonist used, the expression levels of several innate response markers (Fig. 4) were higher for animals infected in the presence of a TLR agonist than animals that received only the dose of *B. pertussis.* The low levels of activation of innate response observed in the *B. pertussis* infected group are indicative of the infective dose used, since infection with higher doses induced significant expression levels of the innate response markers analyzed (not shown). No strict correlation between gene expression pattern and bacterial clearance could be established, since LPS and flagellin induced strong responses at the level of gene induction and neutrophil recruitment to airways (not shown) but showed different effects with regard to *B. pertussis* colonization. This indicates that different mechanisms of bacterial clearance operate triggered by specific pathways and are not part of a single canonical TLR response, since different TLR ligands, although inducing activation of innate response, have different efficiency in the induction of the protective effect. In this context, TLR4-dependent response appears to be the most effective to orchestrate the anti-pertussis effects. According to data obtained in test weight gain, this effect induced by LPS seems not to affect the severity of the disease in the tested conditions. In order to test whether the effect observed with LPS from *B. pertussis* can be induced by LPS from other unrelated organisms, we performed a series of infections using LPS of different origin. As shown in Fig. 3, the number of *B. pertussis* recovered colonies was lower in all cases where LPS was included in comparison to that obtained when the infection was performed only with *B. pertussis* ($P < 0.001$). This indicates that TLR4 activation, irrespective of the source of the agonist, is enough to trigger the anti-bacterial effect that prevents *B. pertussis* colonization. This is coincident with the description of the StIR phenomenon that was achieved by intranasal administration of a *H. influenzae* lysate [6]. In this case, protection of a wide variety of pathogens was achieved, including Gram-positive bacteria (*Streptococcus pneumoniae* and *Bacillus antracis*), influenza virus and *Aspergillus niger* [8, 22]. Since the stimulus used in such case was a complex bacterial lysate, several parallel mechanisms of induction of innate response can be operating and this may explain the broad effects described. In our case, the protective effect is achieved by administration of a specific microbial compound and is shown to be mainly dependent on TLR4 functionality. Several reports have indicated the importance of TLR4 response for eliciting anti-*B. pertussis* immunity [2, 3, 10], and it has been recognized that TLR4-incompetent mouse

strains become infected with lower infective doses than other strains of mice and take more time to control the infections with sublethal doses [2]. Our results indicate that enhancement of TLR4-dependent response at the moment of the infection impairs bacterial colonization, which is in accordance with the aforementioned reports. Moreover, we observed that this activity cannot be substituted with agonists such as flagellin or poly I: C that share part of the LPS signaling pathway (TRIF-dependent and MyD88-dependent cellular activation pathways) [1, 13]. It has been shown that the main cell populations that are TLR competent in murine airways are alveolar macrophages and epithelial cells [18]. Both cell lineages are able to respond to the TLR3, TLR4 and TLR5 agonists that have been used in the present work [7, 23]. However, our results indicate that the effect of impairment of *B. pertussis* colonization is mainly dependent of TLR4 activation. These results may be due to differential effectors triggered by the different stimuli or by differences in the expression levels or the topology of expression of each receptor in key cell populations that take part of the early anti-microbial response. The elucidation of these mechanisms is beyond the scope of the present work and will be further analyzed in future studies.

All together, our results emphasize the role of TLR4 in the early stages of *B. pertussis* infection. This concept not only complements the importance of TLR4 signaling pathway in the pertussis scenario but also represents a new example of the stimulated innate resistance phenomenon.

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